Influence of environmental hypertonicity on the induction of ureogenesis and amino acid metabolism in air-breathing walking catfish (Clarias batrachus, Bloch)

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Effect of environmental hypertonicity, due to exposure to 300 mM mannitol solution for 7 days, on the induction of ureogenesis and also on amino acid metabolism was studied in the air-breathing walking catfish, C. batrachus, which is already known to have the capacity to face the problem of osmolarity stress in addition to other environmental stresses in its natural habitats. Exposure to hypertonic mannitol solution led to reduction of ammonia excretion rate by about 2-fold with a concomitant increase of urea-N excretion rate by about 2-fold. This was accompanied by significant increase in the levels of both ammonia and urea in different tissues and also in plasma. Further, the environmental hypertonicity also led to significant accumulation of different non-essential free amino acids (FAAs) and to some extent the essential FAAs, thereby causing a total increase of non-essential FAA pool by 2-3-fold and essential FAA pool by 1.5-2.0-fold in most of the tissues studied including the plasma. The activities of three ornithine-urea cycle (OUC) enzymes such as carbamoyl phosphate synthetase, argininosuccinate synthetase and argininosuccinate lyase in liver and kidney tissues, and four key amino acid metabolism-related enzymes such as glutamine synthetase, glutamate dehydrogenase (reductive amination), alanine aminotransaminase and aspartate aminotransaminase were also significantly up-regulated in different tissues of the fish while exposing to hypertonic environment. Thus, more accumulation and excretion of urea-N observed during hypertonic exposure were probably associated with the induction of ureogenesis through the induced OUC, and the increase of amino acid pool was probably mainly associated with the up-regulation of amino acid synthesizing machineries in this catfish in hypertonic environment. These might have helped the walking catfish in defending the osmotic stress and to acclimatize better under hypertonic environment, which is very much uncommon among freshwater teleosts.

Keywords: Amino acid metabolism, Ammonia, Clarias batrachus, Environmental hypertonicity, Mannitol, Ornithine-urea cycle, Urea, Walking catfish

The air-breathing walking catfish (Clarias batrachus, Bloch), found predominantly in tropical Southeast Asia, is reported to be more resistant to various environmental challenges such as high environmental ammonia, hypoxic and desiccation stresses. Further, it is reported to be euryhaline, inhabiting fresh and brackish waters as well as muddy marshes, thus facing wide variations of external osmolarity changes; it frequently encounters the problem of osmolarity changes in the same habitat during different seasons of the year, especially in summer when the ponds and lakes dry up, thus compelling this fish to migrate inside the mud peat to avoid total dehydration, and during the monsoon season due to rainfall the water in the same habitat gets diluted.

Several unique physiological and biochemical adaptations have already been reported in this air-breathing catfish with relation to nitrogen, carbohydrate and protein metabolism. These include the presence of a unique functional and regulatory ornithine-urea cycle (OUC) with the capacity of induction of ureogenesis during hyper-ammonia and desiccation stresses. It has recently been demonstrated that the cell volume changes due to osmotic stress can affect the glycogenesis, glycolysis, gluconeogenesis, hexose monophosphate pathway, autophagic proteolysis and also protein synthesis in the perfused liver of this air-breathing catfish. More recently, it has been reported that the walking catfish can survive up to 300 mM mannitol under the laboratory conditions for months without having any mortality, and in situ exposure to higher environmental salinity causes induction of gluconeogenesis. However, no information is available on how the air-breathing

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catfish osmoregulate during exposure to hypertonic environment. Thus, looking at its enormous capacity in challenging the external osmolarity changes, the present study has been undertaken to elucidate the possible induction of ureogenesis with an intention to synthesize and accumulate more urea as an osmolyte, and also the possible changes of amino acid pool by changing the activities of certain key amino acid metabolism-related enzymes in the walking catfish during exposure to hypertonic environment of 300 mM mannitol (equivalent to 300 mOsmol L\(^{-1}\)) for 7 days.

**Materials and Methods**

**Chemicals**—Enzymes, co-enzymes, substrates and mixture of physiological FAA standard, and OPA were purchased from Sigma Chemicals (St. Louis, USA). Other chemicals were of analytical grades and obtained from local sources. Deionized double distilled water was used in all preparations.

**Experimental animals**—C. batrachus, weighing 150±15 g body mass) were purchased from a single source that are bred and cultured in selected commercial ponds. Fish were acclimatized in the laboratory approximately for 1 month at 28±2 °C with 12 h:12 h light and dark photoperiods before experiments when food consumption became normal. No sex differentiation of the fish was done while performing these studies. Minced dry fish and rice bran (5% of body wt) were given as food every day, and the water, collected from a natural stream, was changed on alternate days. Food was withdrawn 24 h prior to experiments.

**Experimental set up**—Ten fishes (pre-weighed) were placed individually in plastic buckets containing 2 L of 300 mM mannitol solution prepared in bacteria-free filtered stream water (pH 7.10 ± 0.05) for 7 days. Another 10 fishes were kept individually in plastic buckets containing 2 L of bacteria-free filtered stream water (pH 7.02±0.06) for 7 days and served as controls. Both the mannitol solution and water from each bucket were replaced with a fresh medium every day at a fixed time after collecting some samples from each bucket for analysis of ammonia and urea concentrations. After 3 and 7 days, five fish each from control and treated buckets were anesthetized in neutralized 3-aminobenzoic acid ethyl ester (MS-222, 0.2 g L\(^{-1}\)) for 5 min, blood samples were collected from the caudal vasculature with a heparinized syringe, tissues such as liver, kidney, muscle and brain were dissected out, plunged into liquid nitrogen and stored at -80 °C. Blood collected from each fish was centrifuged at 10,000 g for 10 min, and plasma were processed\(^4\) for further analysis. All analyses were completed within 2 weeks of collecting the tissues.

**Analyses of ammonia, urea-N and free amino acids (FAAs)**—Amounts of ammonia and urea-N excreted by both control and mannitol-treated fish were measured enzymatically\(^{15}\). Ammonia and urea-N concentrations in different tissues and in blood plasma were also measured by the same enzymatic methods after processing the tissue as described by Saha and Ratha\(^4\).

The concentrations of different physiological free amino acids (FAAs) in tissues and plasma were analyzed in a Shimadzu HPLC (Model LC 20AD) with a post-column derivatization method using o-phthaldehyde (OPA) reagent as a fluorescent dye following the method of Fujiwara \(^{16}\) with certain modifications as detailed in Saha \(^{17}\). In brief, a strong cation-exchange column (Shim-Pack ISC –07 Li, 10 cm long) was used for separation of FAAs. The detector (Shimadzu RF-535 fluorescent detector) was set at an excitation of 365 nm and an emission of 455 nm, and coupled to a data integrator (Shimadzu CR6A) for quantification of the eluted peak areas. The eluting mobile phase was a gradient of buffer A (0.16 N lithium citrate containing 7% methyl cellulose, pH 2.5) and buffer B (0.32 N lithium citrate containing 0.62% of boric acid, pH 10.0), starting with 100% mobile phase A; the flow rate was 0.4 mL min at 0 to 53 min, followed by 0.3 mL/min until the end of the run; the column temperature was 40 °C at 0-40 min, and 50 °C thereafter to 240 min. In the first 40 min the linear gradient progressed to 4% mobile phase B, followed by a linear increase to 10% in 93 min, 30% in 106.7 min, changed to 40% in 106.7 min and was held there until 123 min. The gradient was then increased linearly to 53% mobile phase B in 135 min and held there until 170 min, and finally increased linearly to 100% mobile phase B from 170 to 190 min and held there until 240 min. Hypochloride reagent for on-line oxidation was prepared by adding 0.4 mL of the commercial sodium hypochlorite solution to 1000 mL of the buffer solution (pH 10) containing sodium carbonate (0.384 M), boric acid (0.216 M) and potassium sulphate (0.108 M). The fluorescence reagent was prepared by
adding 2.0 g OPA (dissolved in 1 mL of ethanol), 4 mL of 10% aqueous Brij 35, and 2 mL 2-mercaptoethanol to 980 mL of the above alkaline buffer.

**Extraction procedure**—For the assay of enzymes, a 10% homogenate (w/v) of different tissues was prepared in a homogenizing buffer containing 100 mM Tris-HCl buffer (pH 7.5), 50 mM KCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitor (Roche, Germany) using a motor-driven Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was treated with 0.5% Triton X-100 in 1:1 ratio for 30 min. The homogenate was then subjected to mild sonication for proper breakage of mitochondria and centrifuged at 10,000 g for 10 min. The supernatant was used for assaying the enzymes. All steps were carried out at 4 °C.

For the analysis of FAAs, a 10% homogenate (w/v) of different tissues was prepared in HPLC grade water. Proteins were immediately precipitated out from the homogenate by adding 2 M perchloric acid (PCA) in a 1:1 ratio, followed by centrifugation at 10,000 g for 10 min. The plasma was also treated with 2 M PCA in a 1:1 ratio to precipitate out the proteins, and further processed as above. All these steps were performed at 4 °C. The pH of the resultant supernatant was adjusted to 2.2 by adding a known volume of 0.4 N Li-hydroxide. The supernatant was passed through a Millipore micro filter (0.45 µM pore size) before analysis.

**Enzyme assay**—The enzymes of OUC, viz., carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (ARG) were assayed following the method described by Saha et al. However, for the assay of OUC-related CPS activity, 1 mM of uridine-5'-triphosphate (UTP) was also added in the reaction mixture to inhibit the pyrimidine synthesis-related CPS II activity. It should be noted that the assay method used here for CPS activity does not distinguish between the two different forms of urea synthesis-related enzymes namely CPS I (ammonia- and N-acetyl-L-glutamate-dependent, mitochondrial) and CPS III (glutamine- and N-acetyl-L-glutamate-dependent, mitochondrial). The reaction for all the enzymes was stopped by adding 0.5 mL of 10% perchloric acid mL−1 of reaction mixture after a specific time of reaction, followed by centrifugation to precipitate out the proteins. Citrulline formed in the case of CPS and OTC, citrulline used in the case of ASS, and urea formed in the case of ASL and ARG were measured spectrophotometrically (Varian, Cary 50) in the supernatant and expressed as enzyme activity. All the enzyme assays were carried out at 30 °C. One unit of enzyme activity was defined as that amount that catalyzed 1 µmole of product formed or substrate used h−1 at 30 °C.

Glutamate dehydrogenase (GDH, both reductive amination and oxidative deamination) activity was assayed following the method of Olson and Anfinsen with modifications of substrate (optimal) concentrations. The alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) activities were assayed following the method of Foster and Moon with modifications in substrate (optimal) concentration. All these enzymes were assayed at 30 °C in a UV-visible spectrophotometer fitted with a peltier temperature-controlled unit (Varian, Cary 50) at 340 nm (Em Mγ = 6.22). Enzyme activities were expressed as units g−1 wet wt of tissue and corrected for any non-specific activity in the absence of substrate. One unit of enzyme activity was expressed as that amount which oxidized 1 µmole of NADH or reduced 1 µmole of NAD+ h−1 at 30 °C.

Glutamine synthetase (GS) was assayed by the γ-glutamyl transferase reaction as described by Webb and Brown. One unit of GS activity was expressed as that amount which catalyzed the formation of 1 µmole of γ-glutamyl hydroxamate h−1 at 30 °C.

**Blood sampling and osmolarity measurement**—The blood from each fish was collected with a heparinized syringe from the caudal vein and centrifuged at 10,000 g for 10 min at 0±2 °C for separating out the plasma from blood cells and the plasma osmolarity was measured with a Camlab (Model 200) osmometer using the freezing point depression method.

**Analysis of water content in different tissues**—The water content in cells of different tissues of both control and mannitol–treated fish was determined by oven drying method following Goswami and Saha. Differences with P<0.05 were regarded as statistically significant.
Results

Excretion pattern of ammonia and urea-N by the fish in hypertonic environment—As shown in Fig. 1, the rate of ammonia excretion averaged to 308 μmoles kg⁻¹ body wt h⁻¹ by the control fish during the period of 7 days. Exposure to hypertonic environment (300 mM mannitol, which is equivalent to 300 mOsmol L⁻¹) led to a decrease of ammonia excretion by 2.1-fold within the first day. The decreasing pattern of ammonia excretion rates were maintained in hypertonic environment over the period of 7 days by the catfish with a maximum decrease by 3.3-fold after 5 days.

In contrast, the rate of urea-N excretion, which was averaged to 86 μmoles kg⁻¹ body wt h⁻¹ by the control fish during the period of 7 days, increased significantly by 1.5-fold after the first day of exposure to hypertonic environment with a maximum increase by about 1.8-fold after 6 days (Fig. 1). The ratio of ammonia/urea-N excretion decreased initially from 3.5 to 1.2 within 2 days of exposure, followed by further decrease to about 0.65 from third day onwards and was maintained till 7 days of experimental periods.

Changes of tissue levels of ammonia, urea-N and FAAs in hypertonic environment—The changes in concentrations of ammonia and urea-N in different tissues and plasma of C. batrachus during exposure to hypertonic environment for 7 days are shown in Fig. 2. The concentration of ammonia increased significantly in all the tissues studied (except in brain), and in plasma of fish within 3 days of exposure to hypertonic environment, followed by further increase to about 1.5 to 1.75-fold after 7 days of exposure. Similarly, the concentration of urea-N in different tissues and plasma also increased significantly by about 1.3 to 2.3-fold after 3 days,

Fig. 1—Changes in the rates of excretion of ammonia and urea-N (μmoles kg⁻¹ body wt h⁻¹) by C. batrachus following exposure to hypertonic mannitol solution. Values are mean ± S.E. from 5 observations each. *P <0.001 (Student’s t-test)

Fig. 2—Changes in concentrations of ammonia and urea-N in different tissues (μmoles g⁻¹ wet wt) and in plasma (μmoles mL⁻¹) of following exposure to hypertonic mannitol solution. Values are mean ± S.E. from 5 observations each. P values: a <0.05, b <0.01, c <0.001 (Student’s t-test)
followed by further increase by 1.5 to 3.1-fold after 7 days of hypertonic exposure.

Hypertonic exposure was recorded to cause significant increase in the concentration of total non-essential and essential FAAs in different tissues and plasma of *C. batrachus* (Fig. 3 A-E). In liver, a significant increase in concentrations of different non-essential FAAs was seen after 3 days of exposure to hypertonic environment, followed by further increase of approximately 2.0-fold after 7 days (Fig. 3A). This was mainly attributable to the increase of concentrations of Asp, Gly, Ala, Glu, Gln and Tau. The essential FAAs also increased significantly in liver by 1.3-fold after 7 days of exposure, which was

Fig. 3—Changes in concentrations of different FAAs (µmoles g⁻¹ wet wt) in (A) liver (B) kidney (C) muscle (D) brain and (E) plasma of *C. batrachus* following exposure to hypertonic mannitol solution. Values are mean ± S.E. from 5 observations each. *P* values: a <0.05, b <0.01, c <0.001 (Student’s *t*-test)
mainly attributable to the increase of Thr, Mat, Leu and Arg.

In kidney, the concentration of total non-essential FAAs increased significantly by 1.75-fold after 3 days and by 1.9-fold after 7 days of exposure, which was mainly attributable to the increase of Asp, Ala and Glu, Gly and Gln (Fig. 3B). The essential FAAs also significantly increased by 1.5-fold within 3 days of exposure but with not much of changes after 7 days.

In muscle, the concentration of total non-essential FAAs increased by 1.75-fold after 3 days and by 2.3-fold after 7 days of exposure (Fig. 3C). It was mainly due to increase of Asp, Gly, Ala, Glu, Gln, Pro and Tau. The essential FAAs also increased significantly by 1.50-fold after 7 days of exposure, which was mainly attributable to the increase of Thr, Val, Met and Phe.

In brain, the total non-essential FAAs concentration significantly increased by 1.7-fold after 3 days with a further increase by 1.90-fold after 7 days of hypertonic exposure, which was mainly attributable to the increase of Asp, Gly, Glu, Tau and Gln (Fig. 3D). Likewise, the levels of essential FAAs also increased by 1.95-fold after 3 days of exposure with no further changes after 7 days.

A significant increase in the concentration of total non-essential FAAs was also seen in plasma by 2.6- and 3.9-fold after 3 and 7 days of exposure to hypertonic exposure, respectively (Fig. 3E). This was mainly attributable to the increase of Asp and Gly, Ala, Glu, Gln and Tau. The levels of essential FAAs also increased significantly by 1.90-fold after 3 days with no further changes after 7 days.

The changes in the activities of OUC enzymes in hypertonic environment—The changes in the activities of OUC enzymes in C. batrachus due to exposure to hypertonic environment were studied in liver and kidney (two ureogenic tissues) (Fig. 4). Both in liver and kidney tissues significant increases of activities of CPS, ASS and ASL were observed within 3 days of exposure to hypertonic environment, followed by further increases of activities after 7 days. In liver, the CPS, ASS and ASL activities increased maximally by 2, 1.8 and 7.5-fold, respectively, after 7 days of exposure. In case of kidney, the activities of CPS, ASS and ASL increased maximally by 1.8, 1.9 and 1.67-fold, respectively.

The changes in the activities of certain key amino acid metabolism-related enzymes in hypertonic environment—The activities of 4 key amino acid metabolism-related enzymes, viz., GS, GDH, AST and ALT also increased significantly in different tissues of C. batrachus after 3 days of exposure to hypertonic environment with a further increase after 7 days (Fig. 5). The activities of GS, GDH, AST, and ALT increased maximally by 1.6 to 1.8-fold, 1.6 to 2.0-fold, 1.65 to 1.8-fold and 1.5 to 2.0-fold, respectively, in different tissues.

Changes in plasma osmolarity and tissue water content due to environmental hypertonicity—In situ exposure of C. batrachus in hypertonic environment (300 mM mannitol) led to significant increase of plasma osmolarity by 23 and 30%, respectively, after 3 and 7 days of hypertonic exposure (Table 1). Hypertonic exposure also led to a significant decrease of water content in different tissues within 3 days, followed by further decrease in most of the cases after 7 days.

**Fig. 4**—Changes in the activity (units g⁻¹ wet wt) of different OUC enzymes in the liver and kidney of C. batrachus following exposure to hypertonic mannitol solution. Values are mean ± S.E. from 5 observations each. *P* values: a <0.05, b <0.01 (Student’s t-test) CPS – carbamoyl phosphate synthetase; OTC – ornithine transcarbamylase; ASS – argininosuccinate synthetase; ASL – argininosuccinate lyase; ARG – arginase
Discussion

The role of urea for osmoregulation is well established in marine elasmobranchs\textsuperscript{24}, and to certain extents in marine teleosts\textsuperscript{25}. The crab-eating frog (\textit{Rana cancrivora}), which is one of only a handful amphibians worldwide that tolerate saline waters, is also reported to synthesize and retain urea inside the body by up-regulating the hepatic urea synthetic capacity in hypertonic saline environment\textsuperscript{26}. However, till date no report is available on the role of urea for osmoregulation in freshwater teleosts and more specifically in air-breathing teleosts especially when they are submerged in brackish or saline waters. As reported recently, the \textit{C. batrachus} has a capacity to acclimatize well in high environmental salinity\textsuperscript{14}. In the present study, when \textit{C. batrachus} was exposed to hypertonic environment (300 mM mannitol), the ammonia excretion rate reduced to half with a concomitant increase of urea-N excretion rate by 2-fold, thus causing a reduction in the ammonia:urea-N excretion rate from 3.5 to 0.65. The initial decrease of ammonia excretion in this fish was accompanied by the increase in concentrations of ammonia in different tissues. However, the toxic ammonia was not allowed to accumulate in body tissues to a lethal concentration in fish exposed to hypertonic environment possibly due to possessing various biochemical adaptational strategies to nitrogen metabolism that have been reported in this fish while exposing to different environmental constraints\textsuperscript{2,1}. One of the unique adaptations that it possesses is the presence of a functional and regulatory OUC, and reported to help in detoxification of ammonia to urea under hyper-ammonia stress\textsuperscript{4,6,8}. Further, since mannitol fails to enter into the animal body, it caused dehydration stress to different tissues by exosmosis.

Table 1—Changes of plasma osmolarity and tissue water content in \textit{C. batrachus} following exposure to hypertonic environment (300 mM mannitol). [Values are mean ± S.E. from 5 observations each]

<table>
<thead>
<tr>
<th></th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma osmolarity (mOsmol l(^{-1}))</td>
<td>265 ± 3</td>
<td>325 ± 5* (+23)</td>
</tr>
<tr>
<td>Tissue water content</td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>Liver</td>
<td>78 ± 1.5</td>
<td>70 ± 1.2* (-10)</td>
</tr>
<tr>
<td>Kidney</td>
<td>79 ± 2.0</td>
<td>72 ± 1.8* (-9)</td>
</tr>
<tr>
<td>Muscle</td>
<td>76 ± 2.1</td>
<td>69 ± 1.7* (-9)</td>
</tr>
<tr>
<td>Brain</td>
<td>77 ± 1.9</td>
<td>72 ± 1.8 (-6)</td>
</tr>
</tbody>
</table>

Tissue water contents are presented as percentage of wet mass weight of individual tissue. Percentage increase (+) and decrease (-) of values corresponding to respective controls are given in parentheses.

* \(P\) value significant at <0.05 level compared to respective controls (Student’s \(t\)-test)
thereby resulting in significant decrease of cellular water content in different tissues. The decrease of cell volume/hydration status due to hypertonicity has recently been reported to cause the stimulation of proteolysis in perfused liver of *C. batrachus*\(^\text{12}\). Thus, the increase of ammonia levels in body tissues appeared to be mainly through protein degradation and amino acid catabolism primarily to meet the high energy demand for the osmoregulatory acclimation. Further, the increase of urea excretion rate and higher accumulation of tissue urea levels, observed in hypertonically-treated fish, were mostly associated with the induction of activities of certain key OUC enzymes, viz., CPS, ASS and ASL in liver and kidney tissues of this fish. A 5-fold increase of CPS activity during exposure to 0.9% sodium chloride solution for 14 days\(^\text{27}\) and during exposure to hypertonic saline environment\(^\text{33}\) was reported in the liver of aquatic frog (*Xenopus laevis*). Increase of both ammonia and urea excretion rates were reported in another air-breathing fish, the climbing perch (*Anabas testudineus*) while acclimating to 30% seawater\(^\text{29}\). However, the source of urea in this fish was suggested to be either through arginolysis and/or purine degradation pathway, since the functional OUC was reported to be non-functional in this fish\(^4\). Similarly, in swamp eel (*Monopterus albus*) decrease of both ammonia and urea excretion rates have been reported during exposure to brackish water\(^\text{30}\). Increase of urea level has also been reported in the plasma of carp (*Cyprinus carpio*)\(^\text{31}\) and in anura (*Rana cancrivora*)\(^\text{32}\) while exposing to hyper- and hypotonic mannitol. The decrease in ammonia excretion rate in *C. batrachus*, observed during exposure to hypertonic environment, was probably associated with the enhanced synthesis of urea from ammonia via the induced OUC, thereby leading to stimulation of urea excretion rate and more accumulation of urea in body tissues. Thus, it appears that urea, which is also known to be a good osmolyte, plays a critical role in this fish for cell volume regulation and also to avoid excessive water loss under hypertonic stress.

Liver is the most important organ with relation to nitrogen metabolism and also it is the most important organ governing amino acid homeostasis in fish\(^\text{33}\). Exposure to hypertonic mannitol solution led to significant increase in the levels of various non-essential FAAs (Asp, Gly, Ala, Asn, Glu, Gln and Tau) and also certain essential FAAs (Met, Leu, Trp, and Phe) in the liver of walking catfish. Similar increasing patterns of different FAAs were also observed with minor variations in kidney, muscle, brain and plasma of *C. batrachus* while exposing to hypertonic mannitol solution. One reason of increasing the amino acid pool in different tissues could be associated with the stimulation of protein degradation, which was suggested earlier at least in the liver of this fish under hypertonic stress\(^\text{12}\).

The adjustment of intracellular FAA concentrations in response to osmotic stress has widely been documented among crustaceans as well as other marine invertebrates\(^\text{34–36}\). However, reports on the role of amino acid pool in osmoregulation in freshwater teleosts are scanty. More recently, role of FAAs in osmoregulation has been emphasized in the climbing perch (*A. testudineus*)\(^\text{39}\), swamp eel (*M. albus*)\(^\text{30}\) and in freshwater marble goby (*Oxyeleotris marmorata*)\(^\text{37}\) during acclimation to either sea or brackish water. The basal levels of activity of some of the key enzymes related to amino acid metabolism such as the GS, GDH (reductive amination), AST and ALT were found to be quite high in *C. batrachus* compared to many ammoniotelic teleosts including the gobiid fish\(^\text{38–43}\), which got further stimulated in different tissues (except for AST in liver and brain) under hypertonic stress. Thus, the increasing activity of most of the enzymes, observed in different tissues of mannitol-exposed fish, might have helped in extra synthesis of various non-essential FAAs from ammonia, generated due to protein degradation and amino acid catabolism through the involvement of coupled GDH (reductive amination), which synthesizes Glu by assimilating ammonia with α-ketoglutarate, and GS enzymes to incorporate another molecule of ammonia to Glu forming Gln, and also to other amino acids by transamination reactions. Stimulation of activity of these enzymes was probably associated with higher accumulation of ammonia in different tissues as reported earlier in the same fish under hyper-ammonia stress\(^\text{17,7}\). Thus, it is very much evident that the up-regulation of non-essential FAAs synthetic pathways would be another source of increasing the amino acid pool in this fish under hypertonic stress. Further, the increase of amino acid pool along with the increase of urea concentration, observed in the present study, was associated with the increase of plasma osmolarity, thus counteracting the higher external osmolarity to some extent mainly to avoid the water loss under hypertonic stress.
Interestingly, out of different FAAs, which got accumulated in different tissues of *C. batrachus*, Tau increased maximally during exposure to hypertonic mannitol solution. Tau is a relatively inert molecule, which even at high concentration is not only compatible with the function and organization of macromolecules, but may in addition act as an osmoprotectant counteracting the deleterious effects of denaturing solutes as inorganic ions. The occurrence of Tau synthetic pathway, which constitutes the most predominant amine in most teleosts including *C. batrachus*, is not very clear in fish. Similar increase of Tau concentration was also noticed in this fish under hyper-ammonia stress. Therefore, it is necessary to investigate the occurrence of the Tau synthetic pathway with its physiological significance in this fish.

The mechanism(s) by which different amino acid metabolism-related enzymes and three key OUC cycle enzymes are stimulated in this fish under hypertonic stress is difficult to explain with the available data. More recently, the increase in the abundance of GS and GDH enzyme proteins and mRNA for GDH in mud eel, and GS and GDH enzyme proteins in the juvenile of freshwater marble goby have been reported while exposing to seawater. The more abundance of GS and CPS enzyme proteins and different isoforms of GS mRNAs have also been reported in *C. batrachus* while adapting to high environmental ammonia. Therefore, the transcriptional and translational regulation of these enzymes cannot be ruled out in this fish under hypertonic stress. Another strategy of enzymatic induction could be through changes in the concentration of various effector molecules, and/or by changing the phosphorylation status of the pre-existing enzymes as observed in the case of glycogen phosphorylase and glycogen synthase enzymes in *C. batrachus*, and glycogen phosphorylase enzyme in another catfish, *Amereiurus nebulosus* under aniso-osmotic conditions. Numerous protein kinases have been implicated in metabolic alterations with volume changes in mammalian hepatocytes and in fish chloride cells, and several osmosensing receptors associated with the activation of various kinases have been identified. Molecular crowding may also exert long-term effects, such as cAMP-mediated changes in gene transcription under osmotic stress. However, a thorough investigation requires to be performed to understand better about the regulation of enzymatic activities under osmotic stress in this fish.

In conclusion, the capacity of up-regulating the urea synthesis via the induced OUC resulting to more accumulation of urea in body tissues to defend the osmotic stress in hypertonic environment and also more excretion of urea under hyper-osmotic stress, observed in *C. batrachus*, is uncommon among freshwater teleosts. It resembled more with some aquatic frogs and elasmobranchs. Further *C. batrachus* has the capacity to increase the amino acid pool, by up-regulating the activities of enzymes responsible for synthesis of different non-essential FAAs and probably also by stimulating the protein degradation, is another unique biochemical adaptation that has been observed to acclimatize better under hypertonic environment. Thus, due to possessing these adaptational capabilities *C. batrachus* is able to survive successfully in wide ranges of osmolarity changes in its natural environment.

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